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Classification and Characterization of Hemocytes in *Styela clava*

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Abstract. Viable hemocytes of the common tunicate *Styela clava* are classified into four groups designated as eosinophilic granulocytes, basophilic granulocytes, hyaline cells and lymphocyte-like cells. Eosinophilic granulocytes, actively amoeboid, have large refractive granules that stain with neutral red. Basophilic granulocytes do not stain with neutral red and formed couplets or triplets. Hyaline cells, which often contain phagosomes, have electron-dense small vesicles recognizable only by electron microscopy. Hemoblasts have a characteristic large nucleolus which is visible by light microscopy. Eosinophilic granulocytes and hyaline cells actively ingest yeast particles *in vitro*. This classification simplifies former ones by correlating electron microscopy, with light microscopy, and viable with fixed hemocytes. Clearly viable tunicate hemocytes can be identified by simple methods. We have provided clear and more accurate descriptions which will lessen the controversy often associated with assigning hemocyte functions in immunodefense responses both *in vivo* and *in vitro*.

Introduction

The classification of tunicate hemocytes remains confused, not withstanding Wright's attempt (1981) to devise useful categories. Recent progress in tunicate biology, however, requires a precise correlation between various cellular functions and particular types of hemocytes. *Styela clava*, especially, has been used in investigations of immunological responses including those associated with hemocytes: allogeneic reactions (Raftos and Cooper, 1991); cytotoxic reactions (Kelly *et al.*, 1992a); humoral opsonin (Kelly *et al.*, 1992b, 1993a, b, in press); and the production of cytokines (Beck *et al.*, 1989; Raftos *et al.*, 1991). Humoral lectins (Yokozawa *et al.*, 1986; Harada–Azumi *et al.*, 1987), antibacterial substances (Azumi *et al.*, 1990), and a metallo–protease (Azumi *et al.*, 1991) were studied in another species, *Halocynthia roretzi*.

Although the classification of *Styela clava* hemocytes began early (Ohue, 1936) and the site of hemopoiesis is described (Ermak, 1975, 1976), the literature includes descriptive morphologies with a plethora of terms, but relatively little experimental information uniting structure with function. Previous analyses of hemocytes failed to correlate age, season, and cell behavior in a systematic way, and these variables were not related to the various techniques used for examining them (*e.g.*, staining and fixation versus observation of live cells). Recent molecular and cytological studies focusing on the hemocytes and immune system of *Styela* will reveal a more precise picture of the functional contribution of individual effector cells. But this development depends on a thorough and consistent classification of the hemocytes.

To establish an acceptable and predictable classification scheme, we examined hemocytes from *Styela clava* and correlated the morphological and behavioral characteristics of living hemocytes, and compared appearance of viable cells with those analyzed by light and electron microscopy. Our work offers a strategy for classifying hemocytes in any invertebrate, especially tunicates which are becoming increasingly more important as we decipher the nature of effector cell activity during immune responses.

Materials and Methods

Hemocytes

Hemocytes were harvested by severing the stolons of *Styela clava* after rinsing the outside with 70% ethanol.

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Exuding hemolymph was collected into 0.5 *M* NaCl (NaCl-solution, pH 7.0 by 0.01 N NaOH) in polystyrene tubes; this prevented the nonspecific coagulation of hemocytes and allowed individual hemocytes to be observed. Hemolymph was mixed with the NaCl solution one to one in final volume.

Staining

Hemolymph or hemocyte suspensions in NaCl-solution were loaded onto glass slides. After 10 min, adhering hemocytes were fixed for 15 min and stained with hematoxylin and eosin (H&E). Cold ethanol, cold methanol or 4% paraformaldehyde (0.1 *M* sodium cacodylate buffer, pH 7.0) were used as fixatives, and the morphological preservation was compared. For vital staining, neutral red (NR, 0.01% in final concentration) was added to hemocyte suspensions; 15–30 min later, the hemocytes were loaded onto glass slides and observed.

Correlation of NR-staining with H&E-staining

We photographed NR-stained hemocytes adhering on glass slides, then fixed them for regular light microscopy without moving the slides, and photographed them again under phase-contrast microscopy. After H&E-staining, we found exactly the same cells as in the former two photographs (NR-staining and phase-contrast) to compare their appearance.

Transmission electron microscopy (TEM)

Hemocytes in the hemolymph and inside pharyngeal tissue were examined by TEM. Hemolymph collected into polystyrene tubes was centrifuged (400 × *g* for 5 min) and the pellet fixed. Pieces of pharynx (about 1.5 mm square) were dissected and fixed. Specimens were prefixed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde (0.75 *M* sucrose, 0.2 *M* sodium cacodylate buffer, pH 7.0), then post-fixed with 1% osmium tetroxide in the same buffer. The specimens were dehydrated in ethanol series and embedded in Medcast (Ted Pella, Redding, CA). Propylene oxide was used to infiltrate the resin.

Autonomous fluorescence of viable hemocytes

Hemocytes suspended in NaCl-solution were loaded on glass slides and observed with a Nikon EFD2 fluorescence microscope with blue (420–490 nm)–and ultraviolet (330–380 nm)-illumination.

Composition of hemocytes

Different hemocyte types were counted by light microscopy after H&E or NR-staining, and also by TEM. A sample of hemocytes was taken from 6 animals, and five to ten different viewing fields (110–130 cells in total) from each sample were examined in light microscopy with a 100× objective lens. Five pharyngeal pieces, one each from 5 animals (one TEM-section for each piece), and a hemocyte-pellet from one animal were examined by TEM. About one hundred cells were examined on each section.

Phagocytic activity against yeast particles

Saccharomyces cerevisiae (baker's yeast, type II; Sigma Chemicals, St. Louis, MO) was stained with Congo red and suspended in artificial seawater (approximately 1×10^8 particles/ml), according to Kelly *et al.* (1993a). Hemocyte suspensions in NaCl-solution (100 μl) were loaded on cover slips, and yeast particle suspension (100 μl) was added 5 min later. The hemocytes were incubated for 30 min. After the cover slips were gently rinsed to remove excess yeast particles, 0.01% neutral-red solution was added. Hemocyte types were identified by NR-staining. Types of hemocytes which phagocytized yeast particles were identified.

Results

Light microscopy of hemocytes

Most hemocytes adhered to glass slides, and some of them exhibited amoeboid movement within 5 min. However, many small, transparent cells did not adhere well enough to resist water movement caused by pressure on the cover slip. By phase contrast microscopy, four different types were observed (Table I): (1) hyaline cells, which exhibited significant extensions (15–20 μm in diameter); (2) round cells (basophilic granulocytes, 6–10 μm in diameter), which contained highly refractive small granules and often formed couplets or triplets; (3) amoeboid cells (eosinophilic granulocytes; 8–15 μm in diameter), which contained large granules and exhibited more active amoeboid movement than the other types; and (4) small spherical cells that did not spread (hemoblasts; 4–6 μm in diameter), which contained a small amount of cytoplasm and had a nucleolus clearly visible by light microscopy. The nuclei of hemocytes other than hemoblasts were not visible unless they were spread flat on a glass slide.

Phase contrast microscopy was not sufficient to distinguish all eosinophilic and basophilic granulocytes with certainty. The eosinophilic granulocytes often contained granules as small as those of basophilic granulocytes, and when they were not moving they were just as round as basophilic granulocytes.

We observed large cells that had a hyaline cytoplasm lacking visible granules, but they did contain pigmented or non-pigmented large vacuoles. These cells were also identifiable as hyaline cells because they spread wide and flat. The spreading of hyaline cells was rapid once it began, and these cells did not exhibit active amoeboid movement after they spread.

Table I

Classification and some characteristics of Styela clava *hemocytes*

Type	Eosinophilic granulocytes	Basophilic granulocytes	Hyaline cells	LLC hemoblast
Size	8–15 μm	6–10 μm	15–20 μm	4–6 μm
H&E-staining	Orange	Purple	Very weak purple or pink	Purple
NR-staining	Orange or red-violet	—	Negative or orange at vacuoles	—
Granules in LM	Many refractile	Many small G refractile	—	—
Granules in TEM	Not uniform heterogeneous	Uniform spherical	Small vesicles electron dense	—
Adhesion to glass	++	++	++	±
Phagocytosis	++	+	+++	—
Other characteristics	Active amoeboid movement * Blue fluorecence in red-violet cells	Forming couplets or aggregates	Widely spread (cell fusion?)	Nucleolus visible in LM
Previous classifications	Compartment signet-ring vesiculated morula coarsely granular	Finely granular amoebocyte	Hyaline signet-ring?	Hyaline lymphocyte-like hemoblasts

* Under UV-illumination (330–380 nm).

NR-staining and some characteristics of viable cells

NR mainly stained the cytoplasmic granules of amoeboid cells (Fig. 1). Hyaline cells were usually not stained except for some cases in which small or large cytoplasmic vacuoles were stained (Fig. 1E, F). Two groups of amoeboid cells were positively stained by NR (eosinophilic granulocytes), but the intensity differed. One was stained a dense red-violet, whereas the other was orange (Fig. 1). Both types of cells contained 5–20 large cytoplasmic granules, and they were morula-shaped before starting amoeboid movement. Other granular cells were usually round, unstained by NR, and contained highly refractive small granules (Fig. 1; basophilic granulocytes). Most of the hemoblasts were not stained (Fig. 1), but a few sometimes stained faintly orange.

Following staining with NR, hemocytes were easily distinguished and their characteristic behaviors examined. Both types of NR-positive granulocytes (eosinophilic granulocytes) were active in amoeboid movement, extending many spine-like pseudopodia. NR-negative granular cells (basophilic granulocytes) were less active in amoeboid movement, but extended long pseudopodia. These cells were often observed as couplets (Fig. 1D), triplets or small aggregates composed only of this type of cell, and they did not separate once they came in contact. Occasionally, these cells spread flat after 30–60 min incubation.

H&E-staining

We used three different methods to observe hemocytes with H&E-staining after fixation. Ethanol and methanol significantly modified hemocyte morphology, so only paraformaldehyde fixation was utilized. We observed the same cells with NR-staining and H&E-staining.

Two NR–positive amoeboid cells (red-violet and orange cells) were stained intensely red or pink. Both cells contained various sizes of cytoplasmic granules that stained with eosin, so both cells were classified as eosinophilic granulocytes. The appearance of the cytoplasmic granules was altered by fixation, especially by ethanol and methanol. Cells that were fixed with these agents appeared as vacuolated cells, granular cells, compartment cells or signet-ring cells.

NR-negative amoeboid cells that contain small refractive granules were stained purple with H&E and were designated as basophilic granulocytes. Their cytoplasmic granules were no longer evident after fixation, and cytoplasmic staining was relatively weak after they spread on slides. However, the nuclei of basophilic granulocytes were smaller and more dense than those of hyaline cells.

The cytoplasm of hyaline cells was very thin after spreading so H&E stained them only weakly purple with some orange-stained cytoplasmic vesicles. Phagocytic vacuoles in some of them were stained red. Some hyaline cells contained large cytoplasmic vacuoles and thus appeared as signet-rings. Also their nuclei became larger as they spread.

A few encapsulations of several small cells were observed (small encapsulation; Fig. 2). In addition, small numbers of multinuclear cells were observed in H&E-staining (Fig. 2). These cells spread flat and contained several large nuclei and small vesicles stained with eosin. Finally, hemoblasts stained purple, and their nucleoli became unclear after fixation.

TEM of hemocytes

In centrifuged pellets of hemolymph, we observed five different hemocyte types (Figs. 3, 4): (1) *large cells*

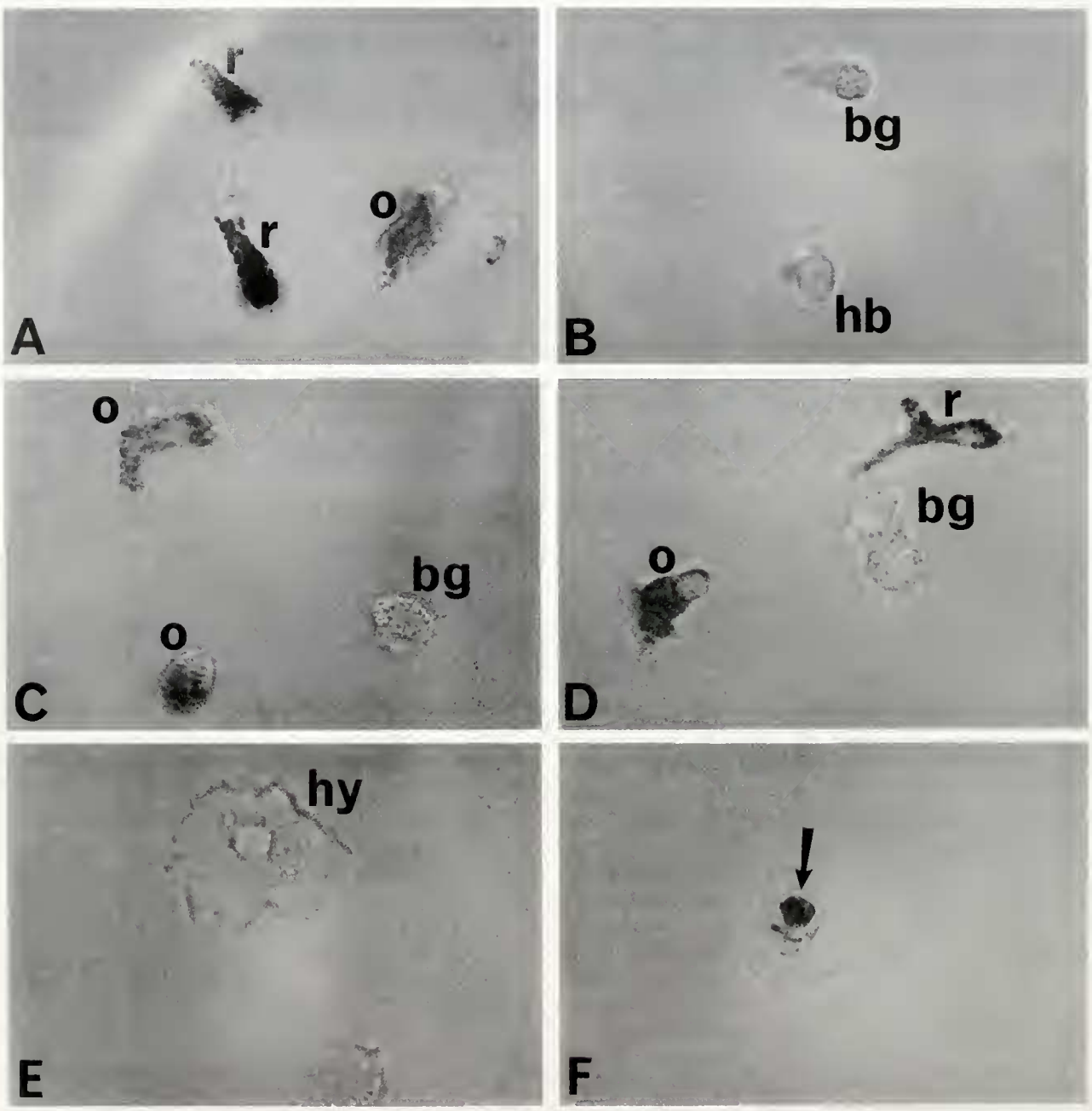

Figure 1. Living hemocytes on glass slides after NR-staining. (A) Eosinophilic granulocytes included two groups of granulocytes that stained in different colors (o = orange and r = red–violet). The sizes of the cytoplasmic granules are variable in each hemocyte. (B) Neither hemoblasts (hb) and basophilic granulocytes (bg) were stained. Nucleoli were evident in hemoblasts. (C) Basophilic granulocytes (bg) contained many refractive granules which were smaller than those of eosinophilic granulocytes (o = orange cells). (D) A couplet of basophilic granulocytes (bg); these were frequently observed. (E) Hyaline cells (hy) spread wide and flat on the glass slide to form a thin cytoplasmic sheet. (F) Some hyaline cells contained granules (arrow) that stained with NR. ×1250

(10–12 μm in diameter: hyaline cells) containing significant amounts of endoplasmic reticulum (ER); (2) *small spherical cells* (5–6 μm in diameter: hemoblasts) with little cytoplasm and large nuclei; (3–5) *three different granulocytes* (6–12 μm in diameter: basophilic and eosinophilic granulocytes) containing abundant cytoplasmic granules. These five types also constituted the entire hemocyte population within the pharyngeal tissue.

The *large cells* contained numerous rough-surfaced and smooth-surfaced ER and also small vesicles (0.1 μm in

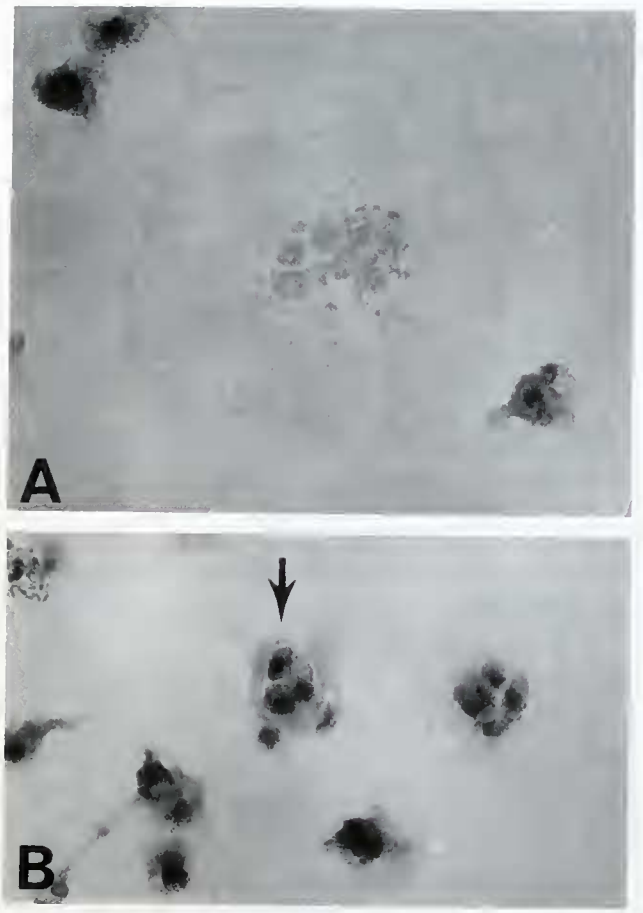

Figure 2. Fixed hemocytes on glass slides with H&E-staining. (A) Multinuclear cells with seven nuclei spread wide and flat. Vesicular structures in the cytoplasm were slightly stained. (B) Small encapsulation (arrow) containing 3–7 small cells; these were sometimes observed in the hemolymph. ×1180

diameter) of high electron density (Fig. 3). A few of these cells contained large vacuoles or phagosomes. Their nuclei often had nucleoli and coarse and uniform euchromatin, although heterochromatin was sometimes observed. These cells corresponded to hyaline cells on the basis of size, phagosomes, and the absence of large cytoplasmic granules.

The *small cells* with little cytoplasm contained mitochondria and small amounts of ER (Fig. 4C). Their nuclei, with characteristic large nucleoli, were usually larger than those of other hemocytes. Chromatin was uniformly distributed and slightly more dense in comparison with hyaline cells. These cells corresponded to hemoblasts in cell size, *i.e.*, little cytoplasm and characteristically large nucleoli.

The *three different granulocytes* (temporarily designated as type 1, 2 and 3 granulocytes according to TEM) had the same nuclear pattern (usually with dense heterochromatin at the periphery and sometimes small nucleoli) but differed in their cytoplasmic granules. *Type 1 granulocytes* (6–10 μm in diameter) contained electron-dense and spherical granules with a diameter range of 0.2–0.5 μm (Fig. 4A). These cells correspond to basophilic granulocytes on the basis of size, the sizes of their cytoplasmic granules (they had smallest granules among granulocytes), and their frequency in the hemolymph. *Type 2 granulocytes* (8–10 μm in diameter) contained irregular-shaped granules that varied in size (0.1–1.3 μm in diameter). The granules contained homogeneous material of intermediate electron-density (Fig. 4B). *Type 3 granulocytes* (8–12 μm in diameter) had cytoplasmic granules that were also irregularly-shaped and remarkably varied in size (0.1–1.5 μm in diameter). These granules were composed of heterogeneous materials—central spheres with high electron density and surrounding material of intermediate electron density (Fig. 4B). Type 2 and 3 granulocytes corresponded to eosinophilic granulocytes on the basis of size, the irregular shape of their cytoplasmic granules, and their frequency of occurrence.

Hemocyte composition

We examined percentages of the various hemocytes in hemolymph by counting each type after NR- and H&E-staining and TEM (Table II). The order of dominance for each type was the same in all cases, but the exact values were somewhat different. The most abundant cells were eosinophilic granulocytes (46.3% in NR-staining); second were the basophilic granulocytes (21.0%); hyaline cells were third (18.5%); and the smallest population was that of the hemoblasts (14.1%).

The percentage of eosinophilic cells in H&E-staining (68.5%) was about the same as the sum of type 2 and 3 granulocytes in the hemocyte pellets observed by TEM (67.8%), but it was larger than the sum of orange- and red-violet cells in NR-staining (46.3%). Many fewer hemoblasts were found in both H&E-staining (2.3%) and TEM (2.0%) than in NR-staining (14.1%). The proportion of hyaline cells ranged from 5.5 to 18.5%, even after the percentages of multinuclear cells and phagocytosis were added. Multinuclear cells (1.2% in H&E-staining) were not found in NR-staining or TEM of pharyngeal tissue.

Autonomous fluorescence of hemocytes

Blue fluorescence was observed in certain granulocytes under ultraviolet-illumination. NR-staining of those fluorescent hemocytes, in the same field of view, revealed that the autonomous fluorescence was from eosinophilic granulocytes which stained in red-violet (Table I). Under blue-illumination, no hemocytes exhibited autonomous fluorescence.

Phagocytosis

Four hemocyte types—*i.e.*, hyaline cells, eosinophilic granulocytes (including red-violet and orange cells in

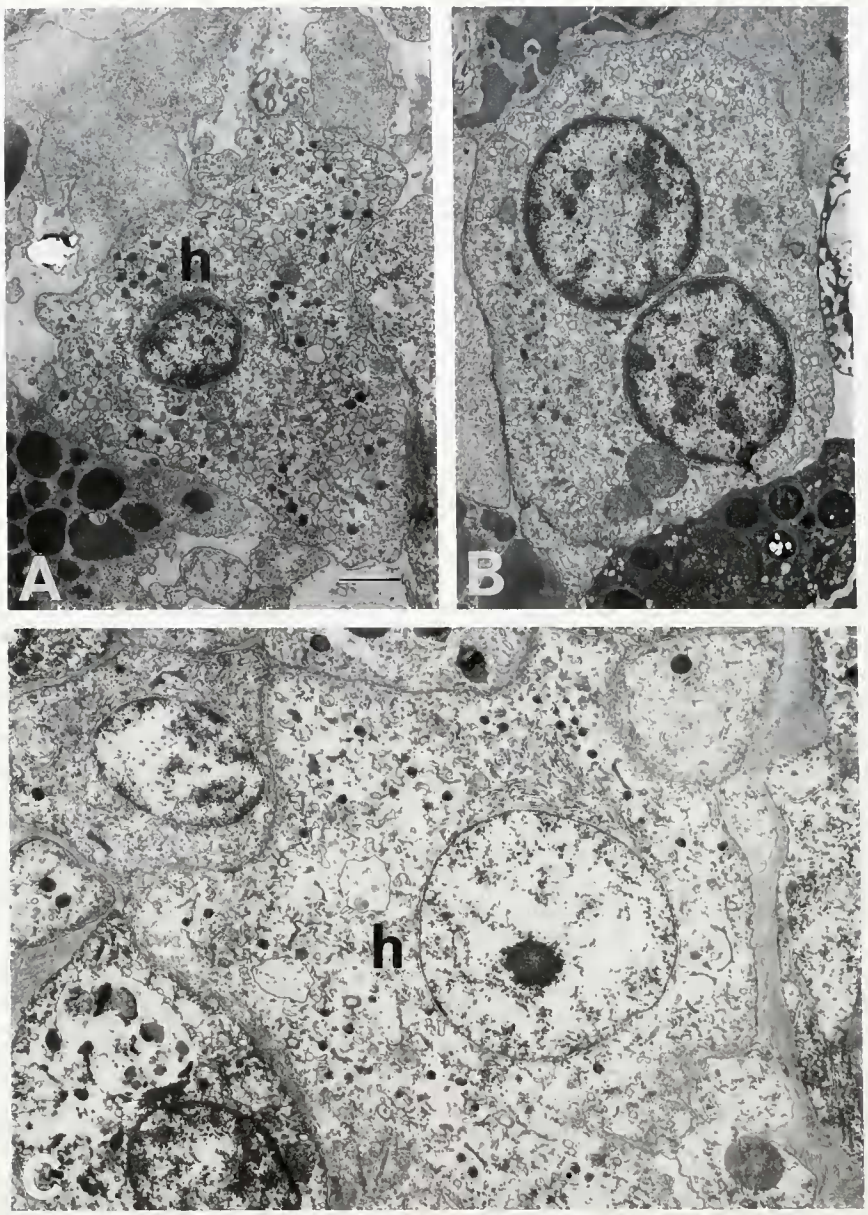

Figure 3. Transmission electron microscopy of hyaline cells and a binuclear cell. (A) Hyaline cell (h) in the centrifuged pellet, with heterochromatin at the nuclear periphery. (B) A binuclear cell in the centrifuged pellet. (C) Hyaline cell (h) in pharyngeal tissue; the nucleus has a large nucleolus and uniform euchromatin. All cells (A, B, C) contained electron-dense small vesicles, numerous vesicular structures, and endoplasmic reticulum. Bar = 1 μm.

NR-staining), and basophilic cells—ingested yeast particles. Among them, hyaline cells and eosinophilic granulocytes had significantly higher activity than basophilic granulocytes (Table III). In the cell population that had ingested yeast particles, hyaline cells (36–42%) were fewer than eosinophilic granulocytes (51–68%), as shown in Table IA. However, phagocytic activity was higher in hyaline cells, because the phagocytic ratios were higher in hyaline cells (32–78%) than in eosinophilic granulocytes (13–35%), as shown in Table IB. Many hyaline cells engulfed 2–5 yeast particles, whereas most eosinophilic granulocytes incorporated only one particle.

Discussion

Classification of hemocytes

Hemocytes from many species of tunicates have been classified by both light and electron microscopy (Ohue, 1936; George, 1939; Endean, 1960; Andrew, 1961, 1962; Overton, 1966; Smith, 1970; Botte and Scippa, 1977;

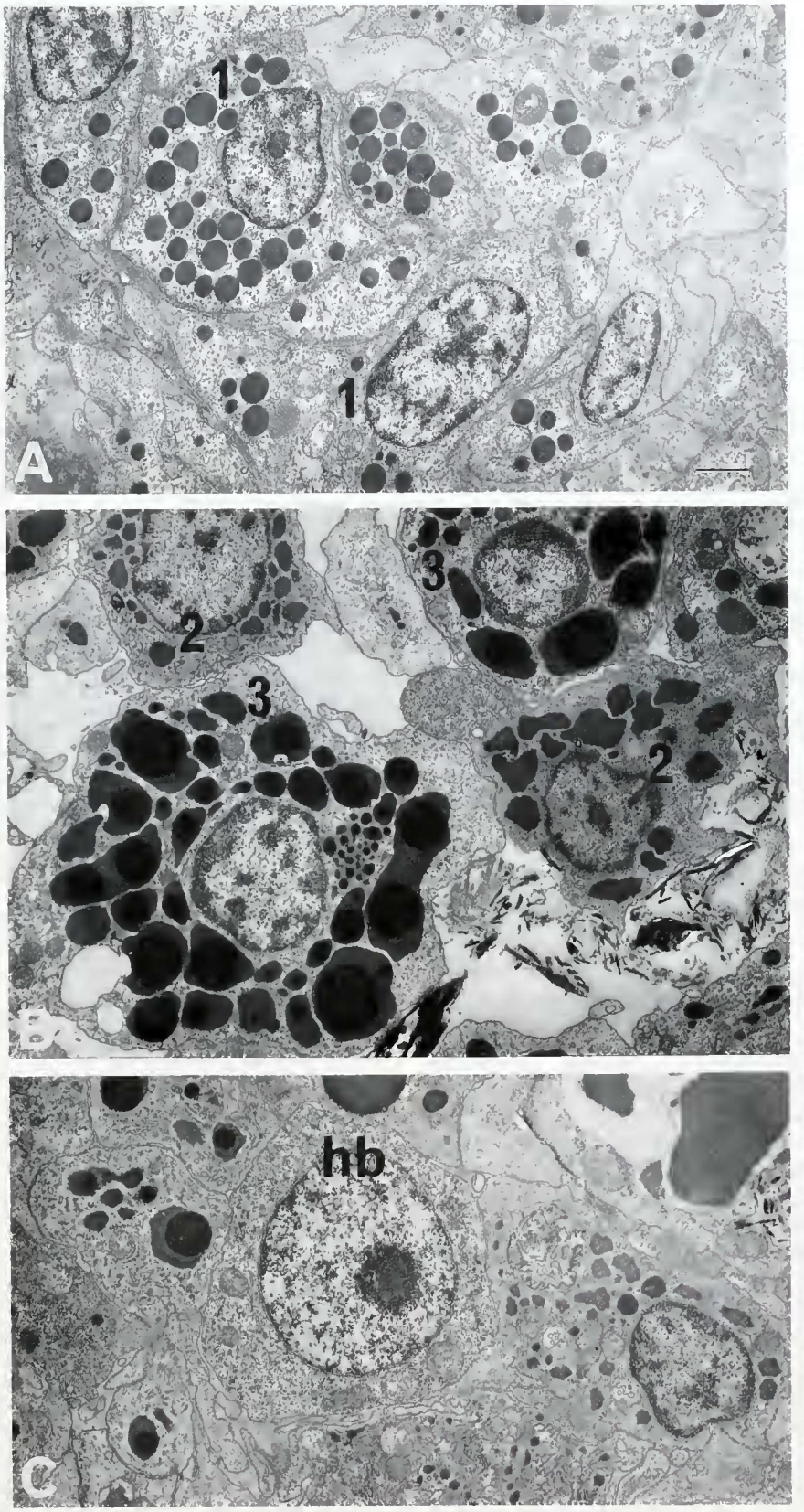

Figure 4. Transmission electron microscopy of hemocytes in the centrifuge pellet of hemolymph. (A) Type 1 granular cells (1 = basophilic granulocytes) containing relatively uniform and spherical granules. (B) Both type 2 (2) and 3 (3) granular cells (eosinophilic granulocytes) containing irregularly shaped granules. The granules of type 3 cells contain electron dense cores. (C) Hemoblast (hb) with little cytoplasm and without cytoplasmic granules, except for mitochondria and vesicles. The relatively large nucleus contains characteristic large nucleolus. Bar = 1 μm.

Table II

Hemocyte composition examined under different conditions

Hemocyte types	Viable cells (NR-staining)	Fixed cells (H&E-staining)	EM (pellet)	EM (pharynx)
Eosinophilic granulocytes	Orange 16.5 ± 5.0%	68.5 ± 5.6%	38.9%	15.8 ± 7.5%
	Red-violet 29.8 ± 8.5		28.9	32.8 ± 11.0
Basophilic granulocytes	21.0 ± 5.1	21.1 ± 4.4	21.0	21.3 ± 7.0
Hyaline cells	18.5 ± 7.9	5.5 ± 2.9	8.0	16.8 ± 3.8
Hemoblasts	14.1 ± 3.2	2.3 ± 2.2	2.0	8.5 ± 6.0
Multinuclear cells	0.0	1.2 ± 1.2	0.2	0.0
Cells of phagocytosis	0.1 ± 0.2	1.4 ± 1.2	1.1	2.3 ± 1.6
No. of individuals examined	6	6	1	5

average ± S.D.

Milanesi and Burighel, 1978; Fuke, 1979, 1980; Rowley, 1981, 1982; Mukai *et al.*, 1990), and in morphological terms, such as vacuolated or granular cells, hyaline cells, hemoblasts or lymphocytes (Wright, 1981), or by *functions* (Freeman, 1964; Fuke, 1980; Fujimoto and Watanabe, 1976; Burighel *et al.*, 1976; Rowley, 1983; Azumi *et al.*, 1990, 1991; Raftos *et al.*, 1990; Raftos and Cooper, 1991). However, the inapplicability of these classifications from one species to the next, and the lack of correspondence between different methods (*e.g.*, light versus electron microscopy) have produced confusion.

Hemocytes of *S. clava* have been classified into morula cells, compartment cells, signet-ring cells, granular amoebocytes, hyaline cells and lymphocyte-like cells (Ohue, 1936; Wright, 1981). But, among fresh and living hemocytes, we observed no signet-ring cells nor any cells with a stable, morula shape. Instead, there were granulocytes that frequently changed their appearance during amoeboid movement. They appeared morula-like when they rounded up, and could be compartment cells or granular amoebocytes after they had become extended and flattened. Fixation, especially with ethanol or methanol, modified hemocyte morphology significantly, and some of the eosinophilic granulocytes and hyaline cells became signet-ring in shape. Therefore, we adopted two cautious guidelines. First, we avoided using such terms as morula, compartment, or signet-ring. Second, we employed no Wright- or Giemsa staining because they require methanol as the fixative. Instead, we preferred to use formaldehyde fixation and H&E-staining.

We identified five different hemocyte types by vital NR-staining and TEM, and four types by H&E-staining of fixed cells. We estimated that the granules of the orange cells in NR-staining contain less dense material, and so correspond to type 2 granulocytes in TEM; similarly red-violet cells in NR-staining correspond to type 3 granulocytes in TEM. The difference between type 2 and 3 cells, or between orange and red-violet cells, is not significant enough to separate them into two cell types. Moreover, both the orange and red-violet cells evidently correspond to eosinophilic granulocytes in H&E-staining. These two granulocytes appear to be similar in amoeboid movement and phagocytic activity. Therefore, we classified both of them into the same group as eosinophilic granulocytes. We suggest that type 2 granulocytes (orange cells) are an earlier stage in cell differentiation than type 3 granulocytes (red-violet cells).

The correspondences between the light microscopical and TEM images of basophilic granulocytes (type 1 granulocytes in TEM), hyaline cells, and hemoblasts were clear on the basis of their morphological characteristics and their frequencies of appearance.

Multinuclear cells were classified as hyaline cells for the following reasons: (1) morphologically multinuclear cells are in all other respects similar to hyaline cells; (2) they sometimes contain large, eosinophilic vacuoles that we assume to be phagosomes; (3) the morphology and behavior of hyaline cells are quite similar to phagocytes type 1 (p1-cells) of *Halocynthia roretzi* (*H. roretzi*), which evidently fuse together and form multinuclear cell sheets (Sawada *et al.*, 1991). But, we have no strong evidence for cell fusion between the hyaline cells of *S. clava*. Moreover, the frequency of multinuclear cells in fresh hemolymph is not clear, because they could be identified only after spreading on glass. Both of these points require further investigation.

In this study, therefore, we have identified four hemocyte types in *S. clava*. (1) Eosinophilic granulocytes contain several refractive vacuoles that appeared red in neutral red vital stain, red by H&E, and exhibit active amoeboid

Table III

Phagocytosis of yeast particles by hemocytes from three different individuals

(A) Composition of hemocytes which ingested yeast particles

Animals	Eosinophilic granulocytes (red–violet)*[1]	Eosinophilic granulocytes (orange)*[1]	Basophilic granulocytes	Hyaline cells	Hemoblasts	Total cells examined
a*[2]	29.2%	22.1%	6.2%	42.5%	0.0%	113
b	41.0	11.0	10.0	38.0	0.0	100
c	41.6	16.8	5.0	36.6	0.0	101

(B) Phagocytosis against yeast particles within each hemocyte type

Hemocyte types	Animals	Ingesting cells	Non-ingesting cells	Total cells examined
eosinophilic granulocytes	a*[2]	19.4%	80.6%	108
(red–violet)	b	30.8	69.2	52
	c	12.9	87.1	101
(orange)	a	16.4	83.6	110
	b	ND*[3]	ND	ND
	c	1.6	98.4	61
basophilic granulocytes	a	7.3	92.7	124
	b	3.8	96.2	53
	c	0.0	100	57
hyaline cells	a	78.0	22.0	100
	b	63.6	36.4	22
	c	32.8	67.2	61

*[1] Two sub-populations of eosinophilic granulocytes different in colors of NR-staining are indicated in parenthesis.
*[2] Animals (a, b, c) in Table A correspond to the animals in Table B.
*[3] No data.

movement and phagocytosis. (2) Basophilic granulocytes contain numerous small granules that do not stain with neutral red, are purple in H&E, and form specific aggregations with the same cell type. (3) Hyaline cells contain fine electron-dense granules in TEM, occasionally contain phagosomes that stain red with neutral red and H&E, and extend into thin circular sheets on glass. (4) Hemoblasts possessed little cytoplasm, large nucleoli visible by light microscopy, but adhere only weakly to glass. Possible correspondence between former classifications are shown in Table I.

Functions and characteristic behavior of each hemocyte type

Phagocytosis, as is well known, is a ubiquitous and important immuno-defense response found throughout the animal kingdom. Hyaline cells exhibited the highest phagocytic activity, and some of them engulfed more than five yeast particles. Eosinophilic granulocytes were less active than hyaline cells, but they accounted for the largest population because of their abundance and active motility.

Hyaline cells were the most likely candidates for effecting encapsulation of larger particles by their ability to spread and form flat sheets and to fuse together into larger multinuclear sheets. Hemoblasts have been referred to as lymphocyte-like cells (Wright, 1981) and as proliferative stem cells (Ermak, 1976). We also observed the characteristically large nucleolus also in viable cells and confirmed their equivalents by light (Wright, 1981) and electron microscopy (Ermak, 1976).

Motility was also an important and definitive, behavioral characteristic. Only eosinophilic granulocytes exhibited active movement. In contrast, the basophilic granulocytes did not separate after once contacting others, which resulted in the formation of couplets or triplets. This behavior continues when augmented, resulting in small aggregates. Similar behavior was also observed on g1-cells of *H. roretzi* (Sawada *et al.*, 1991), and we suggest the presence of common granulocytes that can form specific aggregates within the same cell type.

Correspondence to the hemocytes in other tunicate species

Hemocyte types found in many species have been categorized into several groups by Wright (1981). However, the hemocytes of a single category often include several different types. In addition, certain hemocytes of one species are apparently absent in other species. It would not be instructive to compare only morphological aspects of hemocytes, and only under a single condition, such as in paraffin

sections. Observations of living hemocytes, under different conditions and stained with simple dye, coupled with functional analysis, *e.g.*, of phagocytosis, would be more useful.

In such a manner, we compared the hemocytes of *Styela clava* and *Halocynthia roretzi* which have also been classified in the living state (Sawada *et al.*, 1991), and found interesting correspondences between types. Hyaline cells and basophilic granulocytes were similar to the p1-cells and g1-cells of *Halocynthia roretzi*, respectively, in morphological and behavioral aspects. Hemoblasts, as the candidate for hematopoietic stem cells, may correspond to the ly-cells of *Halocynthia roretzi*, but their function as the stem cells has not been established in either species. Eosinophilic granulocytes seemed to be similar to the v3- and v4-cells of *Halocynthia roretzi* in that refractive vacuoles occupy most of the cell volume, and active amoeboid movement and acidphilic staining occur. But eosinophilic granulocytes of *Styela clava* were evidently more phagocytic. The correspondence between these species of at least two to three cell types may be consistent with their phylogeny.

Acknowledgments

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